

STUDIES ON MALARIAL PARASITES

VI. THE CHEMISTRY AND METABOLISM OF NORMAL AND PARASITIZED
(P. KNOWLESI) MONKEY BLOOD

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IN VITRO

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VI. THE CHEMISTRY AND METABOLISM OF NORMAL AND PARASITIZED (*P. knowlesi*) MONKEY BLOOD*

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The malarial parasite during most of its asexual life cycle inhabits the erythrocytes of the host animal. Hence, a study of the chemical and metabolic properties of the blood of both the normal and infected host furnishes an experimental approach to an understanding of the metabolic processes of the malarial parasite itself. The value of such studies in furnishing information concerning various aspects of malarial plasmodia has been demonstrated by many different workers during the past 5 or 6 years. The first extensive biochemical studies on malarial parasites were those of Christophers and Fulton (1, 2), Fulton and Christophers (3), and Fulton (4) in 1938 and 1939. They reported on the respiratory and glycolytic metabolism of *P. knowlesi*, using a number of carbohydrate substrates, and on the inhibitive effect of antimalarial drugs on the respiration of this plasmodium. Metabolic studies have also been made by Coggeshall (5) and Maier and Coggeshall (6, 7) on three simian species of plasmodia, *P. knowlesi*, *P. inui*, and *P. cynomolgi*, and two avian species, *P. cathemerium* and *P. lophurae*. Their work consisted of respiratory metabolic studies, using several different substrates, and the use of respiratory inhibition as a criteria for the effect of antimalarial drugs. Velick (8) greatly extended the metabolic evaluation of *P. cathemerium* by showing in the canary correlation of cycle growth and nuclear division of the parasites with increased respiration, respiratory quotient, and cytochrome oxidase activity. Perhaps the most comprehensive metabolic study of *P. knowlesi* is that of Wendel (9) in which lactate utilization and respiration were interrelated. The recent studies by Silverman *et al.* (10) and by Speck and Evans (11, 12) on *P. gallinaceum* consider the specific glycolytic enzymes and in addition, relate the effect of the antimalarial drugs, quinine and atabrine, to respiration and glycolysis.

In addition to carbohydrate metabolism, hemoglobin metabolism has been studied. Brown (13) in 1911 was the first to show that hematin was formed

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from the breakdown of hemoglobin. Related to this hematin production is the *in vivo* production of "methemalbumin" which was definitely established by the work of Morrison and Williams (14) and of Fairley and Bromfield (15). That the globin portion of the hemoglobin molecule is utilized by the parasite through the action of proteolytic enzymes as indicated by an increase of the N.P.N. (1) has been postulated by many.

We wish to report here chemical and metabolic observations on monkey blood, both normal and parasitized with *P. knowlesi*, which confirm and extend the findings of earlier workers. Our attention in this study has been particularly directed towards obtaining information and developing chemical procedures pertinent to the perfection of methods for the growth of the malarial parasite *in vitro* (16). The monkey, *Macaca mulatta*, has been employed for these studies and details of the experimental procedures for drawing blood samples, inoculation of malarial parasites, blood staining, and counting techniques will be found described in Paper VII of this series (17). Hematological studies were made concurrently on all blood samples employed. The data are presented under three headings: inorganic composition; respiration; and glucose, lactate, and pyruvate metabolism.

EXPERIMENTAL

Inorganic Composition.—At the outset of our investigations, the need arose for the preparation of a solution similar in inorganic composition and tonicity to that of monkey serum. A search of the literature revealed data only on the concentrations of calcium, potassium, and inorganic phosphate in monkey blood. Calcium values of 2.83 millimols per liter for serum from three normal *rhesus* monkeys are reported by Wats and Das Gupta (18) and a nearly identical figure is given by them for serum from three parasitized animals. An average serum potassium value of 4.68 millimols per liter is reported by Zwemer, Sims, and Coggeshall (19) as a result of their studies on forty-two normal *rhesus* monkeys. Inorganic phosphorus concentrations, ranging from 1.16 to 1.24 millimols per liter of whole blood, are given by three groups of workers; namely, Kerr and Daoud (20), Rapoport and Guest (21), and Wats and Das Gupta (18). The values given by the latter workers are for five parasitized animals.

It has been necessary, therefore, to obtain data on the inorganic composition of *rhesus* monkey blood. We have analyzed both normal and parasitized blood samples for chlorides, potassium, sodium, inorganic phosphorus, bicarbonate, and pH. Chlorides were determined by the method of Wilson and Ball (22), potassium according to the procedure of Kerr (23), sodium by a slight modification of the method of Butler and Tuthill (24), phosphorus by the method of Fiske and SubbaRow (25), bicarbonate by the Van Slyke constant volume gasometric method (26), and pH with the glass electrode of the type described by Claff (27). All chemical analyses were made in duplicate or triplicate.

The results which are given in Table I indicate that the inorganic composition of normal *rhesus* monkey serum and erythrocytes is similar to that of human beings. The predominating cation in the red cell is potassium as is the case in the other two species, birds and human beings, which are susceptible to malaria.

The values of parasitized blood were obtained on samples having from 10.5 to 51.8 per cent (average approximately 30 per cent) of the red cells parasitized. A direct comparison of the composition of whole parasitized blood with normal whole blood is not possible, because of the low hematocrit values encountered in the parasitized samples. A comparison of the serum and calculated red cell values is possible and shows that significant differences occur only in the case of inorganic phosphorus and potassium. The inorganic phosphorus values of parasitized plasma and cells are markedly lower than those of normal blood, the decrease being of about the same magnitude in both cells and plasma. This decrease of inorganic phosphorus in parasitized blood is accompanied by a marked increase in the total phosphorus of the parasitized red cell and is un-

TABLE I

Inorganic Constituents of Normal and Parasitized Monkey Bloods

(Values are expressed as millimols per liter. The number of different animals furnishing blood samples is given in parentheses.)

Inorganic constituent	Whole blood		Plasma		Red cell*	
	Normal	Parasitized	Normal	Parasitized	Normal	Parasitized
Sodium.....	93.9 (5)	127.8 (3)	157.7 (5)	165.2 (3)	8.3 (5)	11.8 (3)
Potassium.....	52.3 (8)	32.2 (7)	5.1 (8)	6.4 (7)	113.1 (8)	121.4 (7)
Chlorides.....	82.5 (4)	88.9 (3)	102.2 (4)	108.6 (3)	55.5 (4)	54.2 (3)
Inorganic phosphate.....	1.5 (16)	1.2 (15)	2.1 (13)	1.4 (15)	0.9 (13)	0.5 (15)†
Bicarbonate§.....	23.6 (2)		28.0 (2)			
pH.....	7.47 (2)					

* Calculated from whole blood and plasma data with the aid of hematocrit values.

† For the fifteen parasitized blood samples, the average hematocrit was 23, while for the thirteen normal bloods, the average was 43.

§ These determinations were kindly carried out by Dr. M. E. Krahle.

doubtedly related to it. This aspect of phosphorus metabolism will be dealt with in a later publication.

The potassium content of the red cell appears to be slightly but significantly higher in parasitized than in normal blood. This is also true of the average plasma potassium levels. However, the potassium values for plasma depend largely on the time when the blood is drawn for analysis. As was shown by Zwemer *et al.* (19), there may be a marked elevation of the plasma potassium level during parasite segmentation and red cell rupture. We have observed similar changes, and in one experiment, a twofold increase in plasma potassium concentration occurred during segmentation. The magnitude and duration of such changes, will, of course, depend upon the degree of synchronous development of the stages of the parasites in the circulating blood.

We have also made determinations on the freezing point of two samples of normal monkey plasma. Values of -0.60° and -0.62° were obtained.

Respiration.—The fact that parasitized blood consumes oxygen at a rate which is rapid in contrast to the low respiration of normal blood has been well demonstrated by previous workers (1–12). The malarial parasite thus presumably obtains some of its energy from oxidative mechanisms in addition to anaerobic glycolytic processes which are the chief source of energy for the host red blood cell. In order to obtain some insight into the merits of various substances as a source of oxidative energy for the growth of parasites and also to obtain knowledge of the oxidative pathway of such substances, we have made some studies on the respiration of the parasitized red cell.

Oxygen consumption was measured at 38.7°C. by the conventional Warburg manometric procedure (28) using 15 ml. conical flasks. Absorption of CO₂ from the blood, especially during the initial stages of an experiment, was found to be slow unless a roll of filter paper protruded from the KOH-containing center well. Even then, it was our practice, before taking readings, to allow 20 to 30 minutes to elapse after placing the vessels in the water bath.

Blood samples were either defibrinated by shaking with glass beads or prevented from clotting by the addition of heparin. No difference in O₂ consumption was found between samples treated in either of these ways or by the use of citrate, though this anticoagulant was not used as routine. The respiration of a sample of parasitized blood drawn from an animal just before the administration of nembutal (32 mg./kg.) was also found to be the same as a sample taken from the anesthetized animal.

The control of pH during measurements is necessitated by two factors: First, the removal of carbon dioxide from unbuffered whole blood causes a pronounced initial alkaline shift in pH. Second, formation of lactic acid by glycolysis during the run causes a progressive acid shift in pH. The choice of a buffering agent against these pH changes must be guided with regard to maintaining an isotonic environment and to the avoidance of interference with metabolic processes. We have as routine employed an isotonic phosphate-Locke solution as a buffering agent. This solution has a pH of 7.3 and the following composition: KH₂PO₄ 0.011 M; Na₂HPO₄ 0.044 M; NaCl 0.070 M; KCl 0.005 M; CaCl₂ 0.001 M. When 1.7 ml. of this solution is added to 1 ml. of whole blood, the pH of the mixture as determined with the glass electrode usually lies within the range 7.6 to 7.2 during the first hour of the measurements. Since more acid pH values are encountered as the run is prolonged beyond 1 hour, we have usually limited readings to this period.

The use of PO₄-Locke as a buffering solution has little effect upon the respiration of parasitized red blood cells as far as we can ascertain. As shown by the data presented in Table II, similar values were observed when whole blood was buffered with either PO₄-Locke solution or with CO₂-free serum of the same pH prepared according to the method of Friend and Hastings (29). To be sure, higher values were observed when untreated serum was the suspending medium, but here it must be remembered a more alkaline pH also prevails. The red blood cells may be separated by centrifuging, washed once with PO₄-Locke solution, recentrifuged, and then resuspended in this solution without impairing their respiration, as indicated by the data in Table II under the column headed "washed cells." In other experiments, we have compared glycylglycine and PO₄-Locke as buffers and have found parasitized cells to respire at the same

rate in each of these mediums. The use of an isotonic (0.11 M) phosphate buffer of pH 7.4 caused a slight inhibition of respiration. Our results on this point are at variance with those of Wendel (9), who concluded that phosphate is contraindicated as a means of controlling the pH of blood containing *P. knowlesi* because of its marked inhibitory effect on respiration.

The release of parasites from the red blood cell by laking has always resulted in a marked decrease in their respiration. In Table II, under the column headed "free parasites," data are presented which show the respiration of a preparation of free parasites to be somewhat less than 50 per cent of that obtained with an equivalent amount of unlaked parasitized red blood cells. This preparation of free parasites was made by centrifuging whole blood, removing the serum, and suspending the cells in an isotonic NaCl solution con-

TABLE II

Respiration of Parasitized Whole Blood, Washed Red Blood Cells, and Free Parasites in Various Mediums

O₂ consumption expressed as mm.³ per hour per flask.

Suspending mediums	Whole blood		Washed cells and glycerol	Free parasites and glycerol
	No substrate	Glycerol		
PO ₄ -Locke.....	90	89	92	42
CO ₂ -free serum.....	94	88		
Serum.....	112	110		

The parasitized blood contained 2.07×10^6 red blood cells per mm.³ of which 34.6 per cent contained parasites. The parasite distribution was 7 per cent rings, 90 per cent trophozoites, 1 per cent schizonts, and 2 per cent gametocytes. Each flask contained either 1 cc. of whole blood or the red blood cells or free parasites obtained from 1 cc. of whole blood. See text for procedure used in preparing washed cells and free parasites.

taining 0.2 per cent saponin. After 5 minutes' stirring at room temperature, hemolysis appeared complete and the suspension was immediately centrifuged in the cold. The packed parasites were then washed once with isotonic saline, recentrifuged, and resuspended in isotonic saline equal in volume to that of the original whole blood. Attempts to obtain higher respiration values on parasites released by other laking agents (lysolecithin, rabbit hemolytic serum) or other suspending mediums were unsuccessful.

The addition of various substrates to parasitized whole blood does not increase its rate of respiration, and it will respire for hours at a constant rate without added substrate. In Table II, data are presented which show that respiration of parasitized whole blood is the same with and without the addition of glycerol. Indeed, the addition of glucose to whole blood has always resulted in a decrease in the rate of respiration, a finding also reported by Wendel (9). In order, therefore, to study the utilization of substrates by the parasite, we

have utilized washed red cell preparations. In Table III are presented data on the respiration of washed parasitized red blood cells in the presence of various substrates. The addition of glycerol, lactate, and glucose to washed parasitized red cells causes a definite increase in their rate of respiration, and their effectiveness is the order of their listing. A slight increase in respiration is observed in the presence of amino acids. Succinate and acetate appeared to be without effect. Also included for comparison in this table are data on the respiration of washed normal red blood cells with and without methylene blue. The normal monkey red cell consumes very little oxygen regardless of the sub-

TABLE III

Oxygen consumption by normal and parasitized washed red blood cells in presence of various substrates, mm.³ per hour per 5×10^9 red blood cells at 38.7°C.

Substrate added	Normal red blood cells	Normal red blood cells and methylene blue	Parasitized red blood cells
None.....	0		95
Glucose.....	6	108	220
Lactate.....		23	293
Glycerol.....		5	299
Amino acids.....		14	119
Succinate.....		11	93
Acetate.....			83

The normal blood had 4.62×10^6 red blood cells per mm.³ The parasitized blood had 1.57×10^6 red blood cells per mm.³, 36.6 per cent of which contained parasites. The parasite distribution was 11.0 per cent rings, 79 per cent trophozoites, 7.5 per cent schizonts, 1.0 per cent segmenters, and 1.5 per cent gametocytes. Each flask contained 1.0 cc. of a washed cell suspension prepared as described in the text, 0.2 cc. of substrate solution (glucose 0.11 M; lactate 0.20 M; glycerol 0.20 M; amino acid mixture approximately 0.15 M; succinate 0.15 M; acetate 0.20 M), and phosphate-Locke solution to make 2.7 cc. total volume. When methylene blue was employed, 0.2 cc. of 0.05 per cent solution replaced 0.2 cc. of the phosphate-Locke solution.

strate present. If methylene blue is added then, as was first shown by Harrop and Barron (30) for other mammalian red blood cells, a marked uptake of oxygen occurs in the presence of glucose. With the possible exception of lactate, other substrates do not appear to be utilized. Parasitized cells thus differ from normal red blood cells in their ability to respire rapidly without the addition of methylene blue and in their ability to utilize glycerol in addition to glucose and lactate as substrates.

The ability of parasitized cells to respire indicates that some respiratory catalyst not present in normal red cells is produced by the parasite. This catalyst would appear to be one containing a heavy metal, presumably either iron or copper since cyanide and carbon monoxide are capable of inhibiting it. Wendel (9) has stated that sodium cyanide (0.01 M) greatly depresses oxygen

consumption of blood parasitized with *P. knowlesi*. We have found that cyanide at a final concentration of 10^{-3} , 10^{-4} , and 10^{-5} M inhibited respiration of parasitized cells by 88, 85, and 16 per cent, respectively. These experiments were performed with the center well of the manometer flask containing KCN-KOH mixtures made according to the directions of Krebs (31). The fact that cyanide does not completely inhibit respiration suggests that the malarial parasite, like other cells, contains a cyanide-insensitive respiratory enzyme of the flavoprotein type.

We have attempted to decide between the presence of a copper or iron respiratory enzyme system in the malarial parasite by the use of carbon monoxide. Though this gas will inhibit both types of enzymes, it is only the iron porphyrin enzymes that are capable of release from CO inhibition by irradiation

TABLE IV
Inhibitory Effect of Carbon Monoxide and High Oxygen Tensions on Respiration of Parasitized Whole Blood

Gas phase	O ₂ consumption
	mm. ³ /cc./hr.
100 per cent O ₂	88
20 per cent O ₂ -80 per cent N ₂	98
5 per cent O ₂ -95 per cent N ₂	112
5 per cent O ₂ -95 per cent CO.....	41

Each flask contained 1 cc. of whole parasitized blood which had 2.51×10^6 total red blood cells per mm.³, 35.8 per cent of them being parasitized. The parasite distribution was 6.5 per cent rings, 82.0 per cent trophozoites, 4.5 per cent schizonts, 0.5 per cent segmenters, and 6.5 per cent gametocytes. Phosphate-Locke, pH 7.3, was employed as a buffer and no substrate was added.

with strong light. As shown in Table IV, a mixture of 95 per cent CO-5 per cent O₂ causes a 63 per cent inhibition of respiration compared to the control flask containing 95 per cent N₂-5 per cent O₂. If the flask containing CO was irradiated, a slight increase in respiration could be observed. In a series of experiments, however, the results were never definite enough to permit a clean cut conclusion to be drawn. On the assumption that the negative results might be due to interference by the hemoglobin present, a few experiments were attempted on parasites isolated by the saponin technique described above. Here, however, the respiration of the material in the control flask fell so markedly during the course of the experiment that again no conclusions were possible. The nature of the cyanide-sensitive respiratory enzyme system of the malarial parasite, therefore, still remains to be determined.

Data are also presented in Table IV which show the adverse effect of high oxygen tension on the respiration of parasites. As the experiment is prolonged,

the effect of a 100 per cent O_2 environment becomes progressively more pronounced. This effect occurs with or without added substrate. A similar observation has been recorded by Silverman *et al.* (10) in their studies on *P. gallinaceum*. In a later paper in this series, we will show the importance of this observation for the successful cultivation of malarial parasites.

Glucose, Lactate, and Pyruvate Metabolism.—The experiments on oxygen consumption presented in the preceding section indicated that the parasitized red cell utilizes glucose. The data presented there, however, gave no clue as to the rate at which glucose was disappearing, nor as to the proportion of glucose being oxidized to that undergoing aerobic glycolysis. Now Wendel (9) has shown that washed monkey red cells parasitized with *P. knowlesi* and suspended in Locke's solution rapidly convert glucose to lactic acid and that the lactate so formed can be further oxidized. In view of this fact and since glucose would undoubtedly constitute the chief energy-yielding material in any culture medium employed, it seemed important to obtain quantitative data on changes in glucose, lactate, and pyruvate concentrations of both normal and parasitized blood maintained under those conditions most nearly approaching physiological.

Blood glucose was determined by the method of Folin and Malmros (32) with slight modifications. Lactic acid was estimated by the method of Barker and Summerson (33) and pyruvic acid by the procedure of Bueding and Wortis (34), a Klett-Summerson photoelectric colorimeter being employed. The samples of blood which were drawn from the leg veins of monkeys were immediately placed in tonometers, equilibrated, and continuously aerated with 5 per cent CO_2 –95 per cent air at $38^\circ C$. The blood cells were kept suspended throughout by gentle rocking of the containers. Initial samples for analysis were withdrawn 10 minutes after temperature and gas equilibration in the constant-temperature room had begun. Analyses were then made at various intervals thereafter. Simultaneously, the oxygen consumption of an aliquot was measured by the procedure described above.

Data from a typical experiment on normal blood are plotted in Fig. 1. The changes that occur may be seen to be fairly linear with respect to time throughout the duration of the experiment. Glucose disappears at a rate of about 0.8 millimols per liter (14.4 mg. per cent) per hour while lactate accumulates at just about twice this rate. Thus, the glucose that disappears can be accounted for nearly quantitatively as lactate. It will be observed that the initial lactate concentration in this blood is 27 millimols per liter. Though this is the highest value we have observed in a sample analyzed directly after removal from an animal, it is not unusual to find the blood lactate elevated due to the hyperactivity of the animal that occurs during handling just prior to bleeding. The average lactate value found from analysis of thirteen different animals, normal and parasitized, is about 12 millimols per liter. The increase in pyruvate concentration during the experiment, though it is nearly twofold, accounts for only a small portion of the glucose that has disappeared. Again, as was pointed

out above, it may be seen in Fig. 1 that normal monkey blood consumes very little oxygen unless methylene blue is present.

Monkey red blood cells thus resemble human red cells. Rapoport and Guest (35) have shown that human blood at 37°C. utilized glucose at a rate of about 15 mg. per cent per hour, converting it nearly entirely to lactate. As in the case then of the human being, the normal erythrocytes of the monkey depend

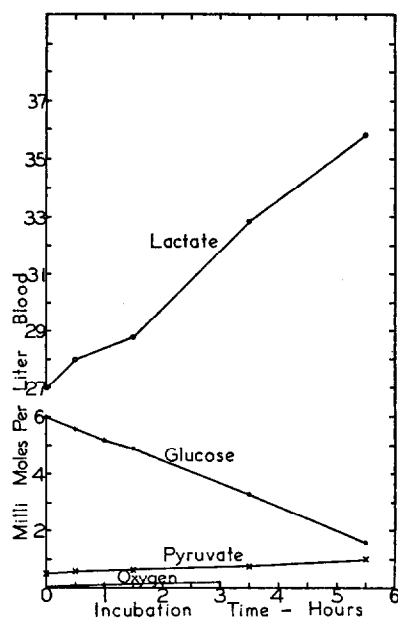


FIG. 1

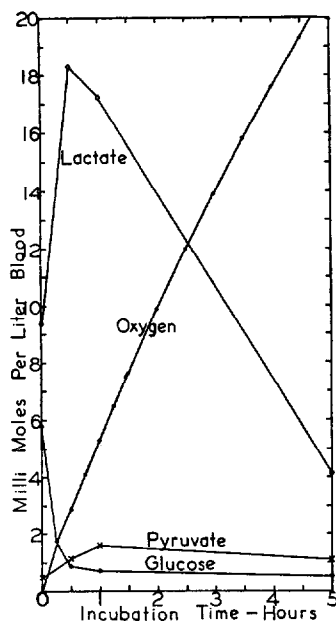


FIG. 2

FIG. 1. Glucose disappearance, lactate and pyruvate changes, and oxygen uptake for normal monkey blood. The erythrocyte count for this blood was $5.50 \times 10^6/\text{mm}^3$.

FIG. 2. Glucose disappearance, lactate and pyruvate changes, and oxygen uptake for parasitized monkey blood. The erythrocyte count for this blood was $3.13 \times 10^6/\text{mm}^3$ with 33.6 per cent of the erythrocytes containing parasites. The differential parasite percentage was: rings 11, trophozoites 85, schizonts 2.5, and segmenters 1.5.

largely on the conversion of glucose to lactate to obtain energy for their maintenance. Regardless then of the ability of parasitized cells to use substrates other than glucose, it is imperative in any culture technique to maintain an adequate supply of glucose if normal erythrocytes are to be present for invasion by parasites segmenting during the culture period. Moreover, in devising culture techniques, it must be recalled that the 100 mg. per cent glucose normally present in blood will suffice for this purpose for not much more than 6 hours at 38°C.

When the malarial parasite is present in the red cell, the picture is greatly changed as can be seen from the representative data presented in Fig. 2. In this experiment, there are only about 57 per cent as many red cells per unit volume as in the normal blood for which data are presented in Fig. 1, but 33.6 per cent of these contain parasites. Here, the glucose has nearly completely disappeared within half an hour, its rate of utilization being of the order of 10 millimols per liter per hour. The apparent failure of the glucose value to drop to zero as the experiment progresses undoubtedly reflects the non-specific nature of the glucose method and is to be attributed to the presence of non-glucose-reducing substances in the blood. About 98 per cent of the glucose that disappears can be accounted for as lactate or pyruvate.

It may be calculated from the data presented that in this experiment each parasitized cell converts glucose to lactate at a rate 68 times that of the normal non-parasitized red cell. As found in other experiments, this rate of conversion varies greatly with the age of the parasite and is roughly proportional to the parasite mass. In general though, it may be stated that the parasitized red cell is 25 to 75 times more active glycolytically than the normal cell. As a rough rule of thumb, therefore, the glucose requirements of a culture of parasitized cells will be increased above that of a unit number of normal cells by 100 per cent for each 2 per cent of parasitized cells present. Thus, whereas 100 ml. of normal blood containing 5×10^{11} red cells will consume at 38°C. 15 mg. of glucose per hour, an identical amount of blood with 2 per cent of the cells parasitized will consume 30 mg. per hour, 4 per cent parasitized cells require 45 mg. per hour, and so forth. Obviously, insofar as the maintenance of satisfactory glucose concentrations during cultivation is concerned, it is advantageous to start with a blood sample containing as few parasites as is consonant with accurate hematological counts.

Unlike the normal red cell, the parasitized cell utilizes lactate. This may be seen from the course of the lactate curve in Fig. 2. During the first half hour, there is a rapid conversion of glucose to lactate which results in a sharp rise in the lactate concentration. After this period, however, there is a progressive fall in the lactate concentration at the rate of 3.3 millimols per liter per hour. This rate of utilization of lactate is about one-sixth the rate at which it was produced from glucose during the initial half-hour period. The utilization of lactate by parasitized cells is dependent on the presence of oxygen; under anaerobic conditions, no lactate is utilized. In Fig. 2, the oxygen consumption of a sample of this blood, determined simultaneously in the Warburg apparatus, is given. During the last 4 hours of the experiment, 15.6 millimols of O_2 per liter of blood were consumed. During this same period, 13.1 millimols of lactate disappeared. The complete oxidation of this amount of lactate would require 39.3 millimols of oxygen. Though the lactate and oxygen determinations were made on samples of blood maintained under slightly different condi-

tions, it seems justifiable to conclude that a large portion of the lactate that disappears is not completely oxidized. The fact that the pyruvate concentration tends to parallel the lactate suggests that pyruvate is a temporary intermediate oxidation product. The fate or nature of other intermediate products is not known.

The ability of parasitized erythrocytes to oxidize lactate indicates that the malarial parasite synthesizes oxidative enzymes not found in the normal red

TABLE V
Lactate Dehydrogenase Content of Normal and Parasitized Red Blood Cells

	Normal blood	Parasitized blood	
		Bottom layer	Feathery layer
Mm. ³ CO ₂ /hr.....	91	97	102
Total cells.....	1.05×10^8	0.94×10^8	0.71×10^8
Parasitized cells, <i>per cent</i>	0	19.2	49.2
Rings, <i>per cent</i>	—	23.5	6.0
Trophozoites, <i>per cent</i>	—	51.0	72.0
Older forms, <i>per cent</i>	—	25.5	22.0
Mm. ³ CO ₂ /10 ⁶ cells/hr.....	0.87	1.03	1.44
Due to normal.....	0.87	0.71*	0.44*
Due to parasitized.....	—	0.32	1.00
Mm. ³ CO ₂ /10 ⁸ Parasitized cells/hr.....	—	1.65	2.04

Each Warburg vessel contained 10 mg. of diphosphopyridine nucleotide (purity 15 per cent), 10 mg. of nicotinamide, 0.4 cc. 0.3 M NaCN, 0.2 cc. 10 per cent K₃Fe(CN)₆ in 0.026 NaHCO₃, 0.4 cc. 0.2 M sodium lactate, 0.2 cc. of the laked cell preparation, and saline bicarbonate (0.025 M HCO₃⁻) to make 3.0 cc. The gas phase was 5 per cent CO₂-95 per cent N₂. The enzyme was tipped from the side arm after equilibration of the system for 15 minutes at 38°C.

* Calculated by multiplying 0.87 (mm.³ CO₂/10⁶ normal cells per hour) by the fraction of normal cells in the sample.

cell. The more rapid conversion of glucose to lactate by parasitized cells than by normal cells suggests also that the parasite synthesizes the enzymes needed for glycolytic processes. It seemed worthwhile, however, to establish definitely whether the parasite was or was not dependent upon the supply of glycolytic enzymes already present in the normal red cell. We have, therefore, assayed normal and parasitized blood for its lactic dehydrogenase content, this being a conveniently studied and representative enzyme of the glycolytic cycle.

Direct measurements of lactic dehydrogenase activity were made using the ferricyanide method of Quastel and Wheatley (36). In this method, ferricyanide is reduced by lactate to

the more acid ferrocyanide which liberates CO_2 from a bicarbonate buffer. The rate of CO_2 liberation as measured by the Warburg manometric procedure is proportional to the lactic dehydrogenase activity. Standard amounts of blood were washed twice with ice cold phosphate-Locke's solution and the cells were laked by the addition of 5 volumes of water; 0.2 cc. of the solution gave suitable activities in the test system employed. Nicotinamide was included in the experimental solution (see below) since preliminary runs indicate that the falling off of CO_2 evolution curves with time could be inhibited by this means, presumably by deceleration of DPN destruction.

The data in Table V were obtained on a sample of normal blood and on a sample of parasitized blood which had been separated into two layers by sedimentation of oxalated whole blood, by the procedure given in paper VII of this series (17). The brown, highly parasitized upper, or "feathery" layer and the more rapidly sedimenting bottom layer were collected separately and the red cells of each batch washed with phosphate-Locke's and laked as described above.

In order to facilitate a comparison, the results obtained, which are given in the top horizontal column of Table V, have been calculated in terms of a unit number of normal and parasitized cells. These derived data, which are also given in Table V, show that parasitized red cells containing mainly half-grown parasites exhibit a lactic dehydrogenase activity two to two and one-half times that of normal cells. It appears also that the lower percentage of rings and the higher percentage of trophozoites in the "feathery" layer is reflected in the amount of enzyme activity observed. This relation between parasite mass and enzyme activity has been borne out in other experiments.

DISCUSSION

The objectives of the investigations reported in this paper have been to obtain information on the chemistry and metabolism of normal and parasitized monkey blood which would furnish a foundation for an attack upon the problem of cultivation of the malarial parasite. It is clear from the results obtained that an adequate supply of glucose will be a prime requisite in any procedure that is employed for cultivation. Fortification of whole parasitized blood with glucose would not appear to be the answer, since the glycolytic rate of parasitized blood is so far in excess of the rate of oxidation of the lactic acid formed that adequate pH control would be impossible. Any successful cultivation procedure would thus seem to demand either the appreciable dilution of whole parasitized blood with a buffered isotonic nutrient medium or provision for the continual addition of glucose and removal of lactic acid from the whole blood by dialysis against a suitable nutrient medium. The data presented here on the inorganic composition of normal and parasitized monkey blood furnish the information needed with regard to the salt components of a suitable nutrient medium. Since the composition of monkey and human blood is nearly identical, such a

basic salt solution should be useful for either simian or human malarial parasites. The data obtained from the respiration studies suggest that glucose, glycerol, and amino acids would be useful energy-furnishing components of any nutrient medium employed for the cultivation of *P. knowlesi*.

The development of these premises for the successful cultivation of *P. knowlesi* is described in the following paper of this series (17).

SUMMARY

1. Normal monkey, *Macaca mulatta*, plasma and red cells are similar in their inorganic composition to those of human beings.

Inorganic phosphate values of plasma and red cells from parasitized monkey blood are lower than normal. Plasma potassium values are higher than normal particularly during segmentation. Other inorganic components of parasitized blood show little variation from normal.

2. Monkey red blood cells parasitized with *P. knowlesi* consume oxygen in the presence of glucose, lactate, glycerol, and amino acids as substrates. Their respiration is inhibited by cyanide, carbon monoxide, and high oxygen tensions. Normal monkey red blood cells consume oxygen at an appreciable rate only in the presence of methylene blue.

3. Parasitized erythrocytes convert glucose to lactate at a rate 25 to 75 times that of the normal monkey erythrocyte. Unlike the normal red cell, the parasitized cell utilizes lactate if oxygen is present. Lactate is utilized, however, at a rate that is only one-sixth that of its production from glucose.

4. The significance of these findings in relation to the problem of cultivation of malarial parasites is discussed.

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